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A tough nut to crack: Intracellular detection and quantification of heme in malaria parasites by a genetically encoded protein sensor

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It is beyond debate that heme plays an immensely important role in major biochemical processes, since it serves as prosthetic group of numerous hemoproteins such as hemoglobin, myoglobin, cytochromes, guanylate cyclase, and nitric oxide synthase. In fact, about 80% of heme occurring in the human organism is produced in red blood cells [1], where it is primarily to be incorporated in hemoglobin. Hemoglobin uses heme as an essential part of the protein's architecture and biological function, i.e. oxygen transport. Apart from its role as a bound factor in such hemoproteins, an increasing number of publications reported about the existence of a significant portion of "labile" heme (also referred to as "free heme", "regulatory heme") in concentrations from 0.1-1.0 µM in cells [2-5]. Today, however, it is clearly not enough known about the maintenance of such labile heme pools which are assumed to ensure bioavailability for proper cellular function under physiological conditions. Under specific pathophysiological conditions an even higher or steadily increasing heme concentration might occur. Thus, a suitable biochemical sensor to detect and quantify heme at a particular time in a particular place is indispensable in order to gain insight into the respective mechanisms. Such specific circumstances, for example, may occur after an infection with malaria parasites [6-8], for which an essential role of free heme in the pathogenesis of the disease was already recognized [8].

Malaria infections affect over 300-500 million people and cause over one million deaths annually, mostly in poor tropical and subtropical countries of Africa [6-8]. 90% of the deaths occur south of the Sahara desert and mainly strike under five-year-old children. A malaria infection starts when a female Anopheles mosquito injects its saliva containing the parasite *Plasmodium falciparum* into the human skin. Destruction of red blood cells is the consequence of this invasion. The degradation of substantial amounts of hemoglobin causes the release of heme within a specialized digestive vacuole. More precisely, in late stages of its development within red blood cells *P. falciparum* digests between 30-70% of hemoglobin. Therefore, the concentration of labile heme is supposed to be significantly increased, however, the exact quantity emerging in the parasite's cytosol was unknown so far.

With respect to progression of malaria infection Niles and coworkers [8] were interested in which way hemoglobin-derived heme undergoes, i.e. whether it is staying in the aforementioned digestive vacuole or escaping to accumulate. Consequently, the quantitative detection of labile heme was the most important analytical question to be solved in this study. In addition, heme detection must be attempted to understand access to heme and its functions regarding its diverse chemical reactions while, at the same time, keeping the cellular damage by heme's redox activity at minimum.

The authors of the respective article in PNAS recently described a genetically encoded protein-based sensor undergoing fluorescence quenching upon heme binding [9]. The sensor was incorporated in the human malaria parasite *P. falciparum*. The original idea of the sensor included a FRET-based system involving the implementation of the fluorescent proteins ECFP (enhanced cyan fluorescent protein) as donor and EYFP (enhanced yellow fluorescent protein) as acceptor (Fig. 1). As a result of a heme-binding protein linker between both fluorescent proteins, heme binding was supposed to lead to a conformational change of the linker resulting in spatial proximity of the proteins and altered fluorescence intensity. The expected fluorescence quenching should appear in a concentration- depending manner enabling the quantitative determination of the surrounding free heme molecules.

P. falciparum Histidine-rich protein II (HRP2) was chosen to function as a suitable heme-binding site of the sensor [9]. Up to 15-18 heme molecules are thought to interact simultaneously with HRP2 in a bis-histidyl mode leading to a dramatic change of the protein conformation upon complex formation [10]. The moderate binding affinity of the interaction seemed promising because a higher affinity could sophisticate the present heme level by acting as a heme scavenger. After recombinant expression of the sensor, spectroscopic studies showed that heme binding to the sensor indeed occured as was described earlier, and the interaction was not significantly changed due to the fusion with ECFP and EYFP (15 heme molecules, K_D 0.25 µM) [9]. In the following, the initial fluorescence-based studies turned out to be a surprise. The sensor was excited at 420 nm expecting an emission maximum at 475 nm (ECFP) before and a maximum at 575 nm (EYFP) after FRET occurred. Excitation in the absence of heme, however, showed maxima at both, 475 nm and 575 nm, indicating that FRET occurred in the absence of heme. Surprisingly, the presence of heme led to stepwise and eventually total loss of the 575 nm maximum upon excitation indicating a lack of photon emission of EYFP. Therefore, the constructed sensor appeared to function as a "turn-off sensor" rather than the originally planned FRET-based sensor [9]. The authors suggested that the observations are a consequence of fluorescence quenching due to heme binding. This seems obvious since heme is a quencher and quenching upon heme binding was described several times before [e.g. 11].

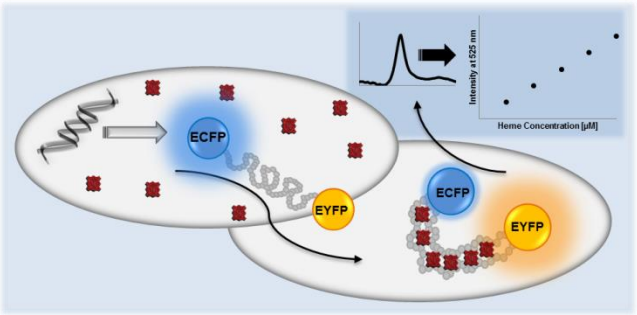

Figure 1. Quantifying of heme (red symbol) by a protein-based intracellular heme sensor. The original idea of Niles and coworkers [9] is based on the conformational change of HRP2 coupled to ECFP (blue) and EYFP (yellow) upon heme binding, i.e. a FRET signal occurs as expected in the presence of heme.

In order to optimize the available heme sensor the authors aimed at using the smallest possible HRP2-derived heme-binding fragment that allows for maximal heme-dependent energy transfer between the fluorogenic proteins. In addition, they intended to reduce the heme-binding affinity of the sensor to reduce also the risk of undetected heme scavenging by the

sensor itself. Several HRP2 fragments were cloned and expressed into the sensor and their impact on quantitative heme detection capacity was studied.

The binding affinity of the shortened sequence stretches did not change significantly compared to the full-length HRP2. However, the stoichiometry acted in direct proportionality to the sequence length. Heme sensing was improved applying the shortened HRP2 fragments. Since the developed heme sensor did not function as a classical FRET-based sensor, the authors defined the new metric E_{DA} that is calculated in the same manner as the FRET efficiency E and represents the effective efficiency of donor-to-acceptor energy transfer. In total a decrease of E_{DA} upon heme application was observed, with E_{DA} being half-maximal in the range of 2-6 µM and exhibiting a greater dynamic range for the shorter heme-binding sequences. A construct including a 49mer HRP2-derived fragment as the heme-binding linker was chosen as the suitable candidate for heme detection in NF54 cells. The robustness of the sensor to e.g. chloride that usually acts as a fluorescence quencher was shown to not significantly affect the obtained results. Thus, the constructed heme biosensor as well as suitable controls, e.g. ECFP and EYFP, were expressed in late-stage parasites. Analysis of the impact of the single compounds in the initial experiments revealed an E_{DA} of 0.71% for the cell-based studies representing a labile heme pool of 1.6 µM [9].

Starting from this point Niles and coworkers aimed to obtain a better understanding of alterations of the labile heme pool in different stages of the disease. During the intraerythrocytic developmental cycle (ICD) *P. falciparum* undergoes various life stages such as the ring-stage, trophozoite stage and the schizonte stage. Fluctuations of the labile heme pool seem probable since a dramatic increase of hemoglobin degradation is usually observed. Using the established sensor the labile heme pool was measured over a time period of 48 hours. Although hemoglobin degradation was significantly increased no major dynamical changes of the labile heme pool were observed in 48 hours. In the next steps the impact of antimalarials on the pool of free heme was explored using chloroquine as an example which used to be the most frequently applied drug for malaria prophylaxes and treatment. However, recently increasing resistances significantly reduce the therapeutic effect of the drug. Therefore it is even more interesting to learn about the molecular mode and mechanism of action in order to be able to develop new antimalarial drugs. In general, it is assumed that drugs like chloroquine interact with heme that is available in high concentrations due to the increased degradation of hemoglobin. The polymerization of heme to form hemozoin might be inhibited, eventually leading to a toxic concentration of the cytosolic heme. After ensuring that the applied sensor did not directly impact the mode of action of chloroquine, different concentrations of the drug were added to parasites expressing the heme sensor. Independent experiments showed a dramatic increase of labile heme when a chloroquine concentration of 60 nM was applied. Yet, determining accurate concentrations of the labile heme pool was problematic due to the fact that chloroquine was able to bind heme leading to a reduced sensor signal [9]. *In vitro* studies showed that chloroquine indeed reduced free heme but in stoichiometric or higher chloroquine concentrations a plateau was observed representing 50-60% of the actual labile heme concentration. Of course a possible underestimation of the occurring concentration should be kept in mind, nevertheless, the sensor allows for a rough estimation of the labile heme pool in the presence of chloroquine. Niles et al. estimated the occurring cytosolic heme concentration in a range of 2.8-7 µM supposing none or only 1:1 chloroquine:heme complexes occur. The obtained results allowed to conclude that the generally observed increase of free heme is a consequence of chloroquine treatment rather than a general cytotoxic effect.

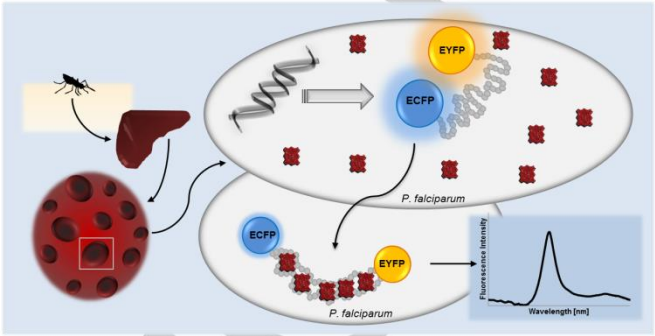

Figure 2. Several species of *Anopheles* mosquitos transmit human malaria. The infection will eventually lead to a significant erythrocytic hemoglobin degradation that is thought to result in an increased pool of labile heme. To determine heme concentrations the "Turn-off sensor" of Niles et al. was expressed in *P. palciparum* and used to quantify heme levels in distinct (patho) physiological situations.

Although the work by Niles et al. represents a very important contribution to the field, it needs to be recognized that genetically encoded heme sensors or other heme detection strategies were reported earlier [12-16]. It has to be noted that in 2015 Atamna et al. still emphasized that methods for measuring free cellular heme independently from total heme were not available up to this time and that knowledge about free heme only came from indirect observations of proteins that transiently bind heme [12]. In the last two years, however, several approaches were published and all these efforts clearly constitute significant progress towards achieving the aforementioned goal. Different subcellular compartments in intact cells were addressed and different solutions for heme detection were tested in these studies. In a first attempt, Atamna and coworkers presented a method based on the reconstitution of apo-horseradish peroxidase (HRP) with heme to form holoHRP [12]. The colorimetric assay used was based on measuring the peroxidase activity upon oxidation of the substrate 3,3',5,5'-tetramethylbenzidine (TMB) by holoHRP. The concentration of free heme in human lung fibroblasts (IMR90) was shown to be appr. 600 nM representing 6% of total heme in these cells. Despite of the successful work done, several drawbacks need to be considered if applying this approach. The amount of total heme was determined by HPLC after lysis of the cells. This procedure could introduce certain sources of interference such as heme degradation during the process and work-up or sticking of heme at proteins and other cellular components apart from the fact that cell lysis deters from analysis of the subcellular heme distribution. Thus, researchers were still interested in the direct measurement of heme in intact cells.

Song et al. introduced the first genetically encoded FRET sensor using the bacterial heme transfer chaperones IsdX1 and IsdC from *B. anthracis* as sensory components [13]. Heme is sandwiched here between the proteins leading to IsdX1-IsdC heterodimerization, which brings the FRET labels ECFP and EYFP attached to the termini in close proximity to allow for fluorescence energy transfer. The study aimed at a spatial and temporal visualization of heme dynamics under living conditions. Thus, the authors first tested whether free heme levels differ in individual subcellular compartments in HeLa cells by specifically targeting an optimized construct containing organelle-specific

peptides to the mitochondrial matrix, the endoplasmic reticulum (ER), and the nucleus. Indeed, it was demonstrated that the heme content in the ER was significantly lower than in the other organelles. This study was extended to other cells types as well, including for example mouse melanoma B16 cells, human HCT116 colon cancer cells, human breast adenocarcinoma cell line MCF-7 and CHO (Chinese Hamster Ovary) cells [13]. Not much later, other groups reported similar approaches employing genetically encoded fluorescent heme sensors to study intracellular heme dynamics and trafficking in different cell types [14, 15]. Hanna et al. studied the labile heme pool in *Saccharomyces cerevisiae* by employing a cytochrome b_{562} derived His/Met-coordinating α-helical bundle (*Holo*-Cyt b_{562}), which was fused to EGFP and mKATE2 (Katushka 2) as fluorescent proteins [14]. The concentrations of free heme found by this approach ranged from 20-40 nM in the cytosol and up to 2.5 nM in the nucleus and mitochondria [14]. A further interesting attempt has been made by introducing genetically encoded hemoprotein peroxidase reporters intended for subcellular targeting in *Caenorhabditis elegans* [15]. Similar to Atamna et al. [12], yet implementing an intracellular detection system, the authors used HRP activity after reconstitution with heme employing HRP variants carrying specific subcellular targeting sequences and tagged with a mCherry fluorescent protein at the C-terminus. This method revealed 433 ± 125 nM of labile heme in HEK293 cells, which were used to test the efficacy of their sensor system [15]. In addition, endogenous heme trafficking as well as transport and distribution of extracellular heme was investigated in more detail, yet is not within aims and scope of this article.

Apart from successful implementation reported in all mentioned literature examples, there are still issues of concern and some aspects of the individual approaches might be regarded with some skepticism [14b]. This can be exemplified with the following: i) How good and stably expressed are such sensors in other cell types. ii) How is the introduction of large, i.e. conformationally demanding, fluorescent labels changing the conformation of the heme-binding/sensing proteins and in this way influencing the heme-binding affinity. iii) What can be said about the quality and intensity of the fluorescence signal occurring in different subcellular compartments and organelles versus the cytosol, just to mention the most important questions here.

In this respect, the presented report by Niles et al. as well as each of the other studies mentioned above represents great advance towards solving the problem of intracellular heme detection and visualization. However, in the long term the pros and cons of the individual strategies, their implementation and applicability in a broader application range need to be assessed and critically evaluated as well.

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Keywords: heme • heme binding • heme biosensor • fluorescence quenching • malaria parasite

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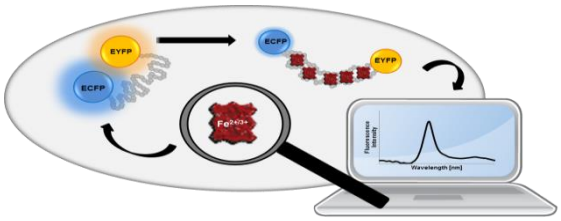
HIGHLIGHT



A genetically encoded protein sensor based on HRP2 was used to detect heme in the human malaria parasite *Plasmodium falciparum* using fluorescence-quenching after heme binding. Protein labels ECFP and EYFP were attached to the termini to allow for a FRET signal as the initial detection parameter before heme binding. The sensor was eventually used to investigate alterations of the labile heme pool during the parasite's life cycle as well as under the influence of the drug chloroquine.

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